ORIGINAL ARTICLE

Age-specific reference intervals for Prostate-Specific-Antigen (PSA), its isoform-[-2] pro-PSA (p2PSA) and prostate health index in Indian males

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Abstract

Background: Studies have shown that there are racial and age-group differences in Reference Intervals (RI) of total Prostate-Specific Antigen (tPSA), its isoform [-2] pro PSA (p2PSA) and % p2PSA, Free PSA (fPSA) and %f PSA, and Prostate Health Index (PHI), all of which have been used for screening and diagnosis of prostate cancer. *Aim and Objectives:* To establish age-specific Reference Interval (RI) for these markers in Indian males. *Materials and Methods:* Four hundred and six subjects with tPSA ≤ 4ng/ml and negative transabdominal prostate ultrasound findings formed the study group in whom the markers tPSA, p2PSA, fPSA were estimated and %p2PSA, %fPSA and PHI were calculated. *Results:* About 98 (24.1%) were ≤ 50 years, 182 (44.8%) were between 50 -59 years and 126 (31%) were ≥ 60 years. The mean of tPSA, fPSA, p2PSA and PHI in ≥ 60 years age group were significantly more than ≤ 50 years age group across all percentiles. The overall RI (2.5^{th}- 97.5^{th} percentiles) for p2PSA, % p2PSA and PHI were 1.29 - 12.52 pg/mL, 0.65 - 3.82 and 8.08 - 66.42 respectively. Though there were some changes in the RI as the age increased, the 90% confidence intervals of the groups overlapped with one another. *Conclusion:* There is need for establishing and validating the age specific RI in healthy subjects belonging to a particular race and ethnicity for any biomarker.

Keywords: Reference Intervals**,** Prostate-Specific Antigen (PSA), [-2] pro-PSA (p2PSA), Prostate Health Index (PHI)

Introduction

The incidence of prostate cancer (PCa) in India is showing an increasing trend and recent epidemiological studies report an incidence rate of 9-10/100,000 population accounting for the fifth highest incidence rate among males in India in 2016 [1, 2]. Estimating serum total Prostate-Specific Antigen (tPSA) remains the most commonly used diagnostic marker for PCa, along with Digital Rectal Examination (DRE) and other imaging modalities [3]. Routine testing of serum tPSA has also been used for screening of PCa, which has helped in the early detection of PCa and has reduced the mortality rates associated with PCa [4-5]. Such screening with serum tPSA however, has also been reported to be associated with unnecessary biopsies, over diagnosis, and over-treatment [4]. Hence, the scientific community is in constant search for more robust tumour biomarkers with better specificity and sensitivity or which can increase the specificity along with tPSA testing. Some of such markers which have been studied in recent times for their utility in screening, diagnosis, and prognosis of PCa include its isoform, [-2] proPSA (p2PSA), %p2PSA, Free PSA (fPSA) and Prostate Health Index (PHI) [6].

The tPSA estimated in the serum is a serine protease, part of a family of kallikrein-related peptidases. In the blood, the majority of PSA (nearly 70 to 90%) is bound with other serum protease inhibitors like α_1-antichymotrypsin and a minor portion (approximately 10-30%) exists as free fPSA [6-7]. Most of the instruments available currently measure the tPSA including both the free and bound PSA. The fPSA exists as three isoforms in the serum – pro-PSA, intact PSA, and benign PSA [8]. The pro-PSA also exists in multiple forms in the serum, and the predominant isoform that is found in tumor extracts is p2PSA [8]. Moreover, studies that did histological analysis of prostate specimens have shown that p2PSA is increased in the peripheral zone and is undetectable in the transition zone which showed that this isoform is more cancer-specific than tPSA [9]. Consequently, most of the studies across the world have focused on the estimation of p2PSA with %p2PSA, fPSA, and %fPSA for their usefulness in the complete management of PCa from screening, diagnosis, prognosis, and response to treatments [8, 10]. Since PCa is associated with higher tPSA and p2PSA and a lower fPSA, studies have included all three parameters in a single index -the Prostate Health Index (PHI) which is calculated using the formula (p2PSA/fPSA) × $\surd$ tPSA) [11]. Studies [10, 12] have shown that both %p2PSA and PHI can accurately predict adverse pathological outcomes in radical prostatectomy specimens including outcomes like upgrading of the Gleason scoring or ⩾ pT3 cancer, positive surgical margin, high-risk disease, or seminal vesical invasion in patients who had clinically organ-confined PCa and were undergoing radical prostatectomy.

However, despite the utility of these markers, many studies [13-15] done in various parts of the world have shown that there are ethnic, racial, and age group differences in the biological Reference Intervals (RIs) of these markers including that of tPSA [16]. Most of the research studies have measured the biomarkers- p2PSA, % p2PSA, and PHI in patients with tPSA values in the "grey zone" that is between 4 to 10 ng/mL and in patients with tPSA levels >10 ng/mL [17-22]. Considering all these facts, measuring these markers in healthy populations and not in just those with abnormal tPSA, and establishing age-group-specific RI is the need of the hour.

Hence the objective of this study was to establish age-specific RI for the PCa markers- p2PSA and % p2PSA, fPSA and % fPSA, and PHI along with tPSA in the Indian male population belonging to this part of the country.

Material and Methods

This study was conducted over 4 years from early 2016 to mid-2019 at a reputed tertiary care oncology hospital in Bangalore, Karnataka which is accredited by various national and international agencies, and the laboratory services being an integral part of the hospital, are also independently accredited. The study was approved by the Institutional Ethics Committee for Clinical Studies (vide letter dated 29/02/2016 bearing the number 001/02-16) and we certify that the study was performed following the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Patient selection and evaluation

During the study period, all subjects who were apparently healthy, and had undergone tPSA testing as a part of their regular health check up and whose tPSA values were ⩽ 4 ng/ml and consented to be part of the study (n = 1123) were initially

recruited. Subjects who had a prior history of PCa, acute or chronic prostatitis, active urinary tract infections, or were on medications that might affect tPSA measurement were excluded from the study. To further ensure that the study group comprised of subjects who did not have any prostate-related pathologies that might affect the values of these PCa markers, only those who fulfilled the inclusion criteria of negative transabdominal prostate ultrasound findings formed the final study group (n = 406). The remaining subjects either did not undergo prostate ultrasound or had some findings related to the prostate gland like changes in the prostate size, presence of nodules, asymmetry of the outer glands, increased blood flow signals in the glands, unclear boundaries between the inner and outer glands or any other abnormal morphology detected during routine transabdominal ultrasound as a part of their health checkup. Clinical & Laboratory Standards Institute (CLSI) guidelines [23], recommend that the minimum number of reference subjects should be 120 for establishing reference intervals. Hence, in this study, the sample size for each of the three age groups was to be a minimum of 120 subjects, however, we were not able to meet the minimum numbers in the age group less than 50 years, and exceeded the minimum numbers in the older age groups.

Sample collection

Blood samples were drawn using standard aseptic precautions in blood collection evacuated tubes manufactured by Becton Dickenson Company, as specified by the kit manufacturer. The sera obtained post-centrifugation was used for the estimation of tPSA immediately and the remaining sera were aliquoted, labeled, and stored at -80^{0}C until analysis of the other markers.

Biochemical analysis of markers

All parameters were estimated in the Beckman Coulter Access-2 Immunoanalyser using the hybritech kits meant for the respective parameter. The parameters tPSA (ng/mL), fPSA, and p2PSA (pg/mL) were estimated and % p2PSA [% of (p2PSA/fPSA)], % fPSA [% of (fPSA/tPSA)], and PHI [(p2PSA/fPSA) $\times\surd$ tPSA] (11) were calculated.

Principles of the biochemical assays

The assay principle of all the markers was the same–two-site chemiluminescent immune-enzymatic (sandwich) principle. The analyte in the sample binds to the immobilized monoclonal anti-analyte on the solid phase and the monoclonal anti-analyte-alkaline phosphatase conjugate reacts with other antigenic sites on the analyte. Materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. When the chemiluminescent substrate is added, light is generated which iss measured with a luminometer. The intensity of the light produced is directly proportional to the concentration of the respective parameter in the sample. The concentration of the analyte is determined from a stored, multi-point calibration curve. Quality assurance of the results was done by using trilevel quality controls from third-party control providers for tPSA, and kit controls for p2PSA and fPSA on the day the samples were processed. The in-house precision check was done for all the parameters using multilevel controls to get a minimum of 20 points for each level and the calculated coefficient of variation was less than 5% for all the parameters.

Statistical analysis

Data was entered into a Microsoft Excel data sheet and SPSS 22 version (IBM SPSS Statistics, Somers NY, USA) software was used for analysis. Grouped data were expressed as frequency and percentage. Continuous data were expressed in Mean ± Standard Deviation (SD) and percentiles. ANOVA test was used to test the significance of mean differences in different age groups. A p-value less than 0.05 was considered statistically significant. The Box and Whisker plots were used to present the continuous data. The calculation of the Reference Interval (RI) was done by non-parametric methods following the EP 28-A3c guidelines given by CLSI [23]. Briefly, this involved a preliminary inspection of the data using a quantile-quantile graph and a histogram to check for any skewness and appropriate distribution. This was followed by inspecting the data for outliers and finally the values at the 2.5^{th} and 97.5^{th} percentiles were used as the lower and upper limits respectively.

Results

Age groups

All the subjects (n=406) were stratified into three groups based on age: less than 50 years, 50 to 59 years, and more than 60 years. In this study, the maximum 182 (44.8%) subjects were in the 50 – 59 years group followed by the more than 60 years group 126 (31%) and the least number of subjects were in the less than 50 years age group 98 (24.1%) (Table 1).

Table 1: Distribution of subjects in different age groups

Age Group	Number of subjects (%)
</=50 years	98 (24.1%)
50-59 years	182 (44.8%)
>/=60 years	126 (31.0%)
Total	**406**

Table 2 gives the means, standard deviation of the mean, and percentiles for selected frequencies for tPSA, fPSA, % fPSA, p2PSA, % p2PSA, and PHI in the different age groups. Value of *p* was <0.001 for all the parameters except % p2PSA (Table 2). The mean of tPSA, fPSA, p2PSA, and PHI in the >60 years age group were significantly more than <50 years age group and it showed the same trend across all the percentiles. There was no significant difference in % p2PSA across the different age groups ($p > 0.05$).

Figures 1 (A to G) and Figures 2 (A, B) show the box plots and curves, representing the distribution of the biomarkers in different age groups. The curves of tPSA, fPSA, p2PSA, and PHI showed the highest peak in the >60 years age group increasing gradually from the < 50 years age group (Table 2, Figures 2A and B). The % fPSA curve for healthy males showed a downward trend decreasing with age ($p < 0.05$). The curve of % p2PSA showed a dip in the age group 50 to 59 years with a peak for >60 years age group.

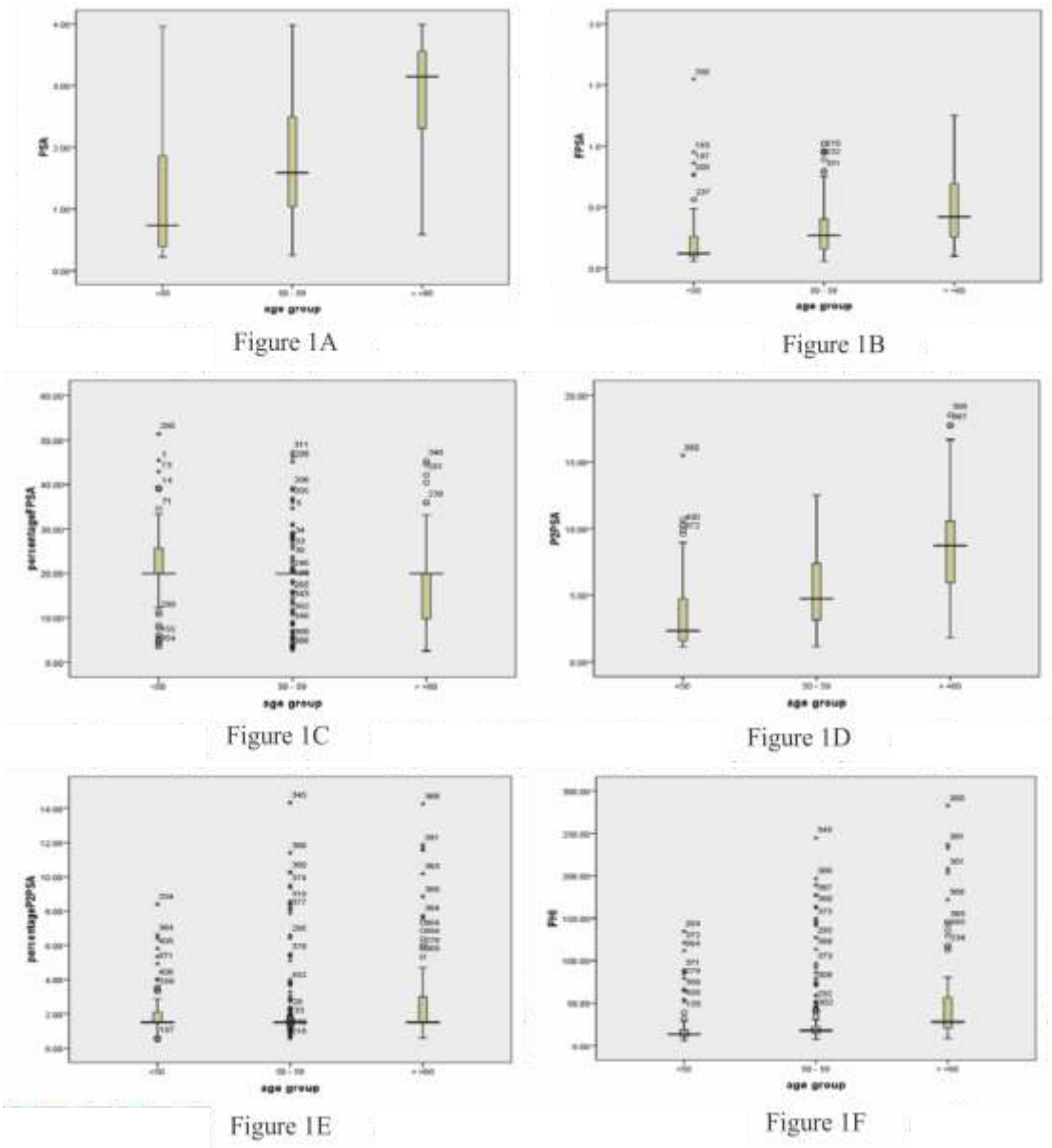

Figure 1: Serum levels (1A) PSA, (1B) fPSA, (1C) %fPSA, (1D) p2PSA, (1E) %p2PSA, (1F) PHI, in healthy males among the different age groups. Boxes and horizontal lines indicate interquartile range

Table 2: Mean ± Standard Deviation (SD) for selected percentiles of all the biomarkers in different age groups

Biomarker	Age group	Number of subjects	Mean ± SD	Percentiles						
				5th	10th	25th	50th	75th	90th	95th
tPSA (ng/mL)	Age (years)	406	1.97 ± 1.17	0.29	0.39	0.98	2.02	3.03	3.58	3.72
	< 50	98	1.17 ± 1.02	0.27	0.28	0.39	0.73	1.86	2.91	3.16
	50 - 59	182	1.77 ± 1.00	0.31	0.46	1.04	1.59	2.49	3.31	3.53
	⩾ 60	126	2.90 ± 0.85	1.21	1.47	2.31	3.15	3.56	3.78	3.96
	p	<0.001*S								
fPSA (ng/mL)	Age (years)	406	0.34 ± 0.24	0.08	0.092	0.15	0.26	0.45	0.698	0.82
	< 50	98	0.21 ± 0.22	0.072	0.08	0.096	0.119	0.26	0.45	0.68
	50 - 59	182	0.31 ± 0.21	0.081	0.091	0.158	0.269	0.402	0.563	0.766
	⩾ 60	126	0.47 ± 0.25	0.136	0.191	0.256	0.418	0.692	0.792	0.902
	p	<0.001*S								
%fPSA	Age (years)	406	19.31 ± 8.32	4.15	6.42	19.74	20	20	28.68	34.87
	< 50	98	21.70 ± 8.50	5.168	10.86	20	20	25.64	31.184	39.05
	50 - 59	182	19.46 ± 7.59	3.822	7.11	20	20	20	28.13	32.49
	⩾ 60	126	17.23 ± 8.71	3.942	5.35	9.75	20	20	28.116	33.662
	p	<0.001*S								
p2PSA (pg/mL	Age (years)	406	5.92 ± 3.66	1.38	1.58	3	5.245	8.67	10.68	11.94
	< 50	98	3.53 ± 2.76	1.28	1.34	1.59	2.33	4.74	7.79	9.75
	50 - 59	182	5.32 ± 3.01	1.4	1.58	3.12	4.74	7.38	10.26	10.89
	⩾ 60	126	8.62 ± 3.46	3.62	4.27	5.94	8.73	10.56	12.36	15.26
	p	<0.001*S								
%p2PSA	Age (years)	406	2.29 ± 2.11	0.756	1.21	1.5	1.5	1.975	4.158	7.374
	< 50	98	2.01 ± 1.30	0.75	1.27	1.5	1.5	2.11	3.5	5.18
	50 - 59	182	2.22 ± 2.15	0.91	1.22	1.5	1.5	1.59	3.87	8.14
	⩾ 60	126	2.60 ± 2.50	0.7	0.94	1.5	1.5	2.98	5.77	7.95
	p	0.096 NS								
PHI	Age (years)	406	33.68 ± 41.71	8.99	10.19	13.33	18.31	28.3	77.04	134.97
	< 50	98	21.74 ± 25.06	7.75	8.7	10.44	13.29	18.31	53.57	86.74
	50 - 59	182	31.52 ± 40.87	9.88	10.38	14.76	17.65	22.2	73.5	145.42
	⩾ 60	126	46.07 ± 49.56	11.49	14.34	20.07	27.9	56.77	108.99	151.13
	p	<0.001* S								

*PSA= Prostate Specific Antigen; fPSA: free Prostate Specific Antigen; %fPSA [% of (fPSA/tPSA)]; p2PSA = [-2] proPSA; %p2PSA= % of (p2PSA/fPSA); %fPSA= % of (freePSA/tPSA); PHI =Prostate Health Index= [(p2PSA/fPSA) ×√ tPSA]; *p-value is significant*

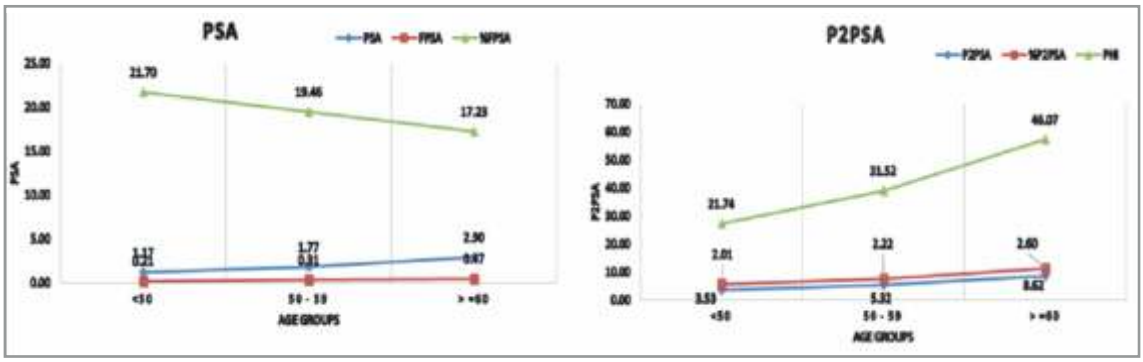

Figure 2: The age-related distribution curves for the median values of (2A) PSA, fPSA and %fPSA, (2B) p2PSA, %p2PSA and PHI

Age-group specific reference intervals for p2PSA, %p2PSA and PHI

The biological RI (2.5th to 97.5th percentiles) for the markers- p2PSA, %p2PSA, and PHI in the overall population were 1.29 to 12.52 pg/mL, 0.65 to 3.82, and 8.08 to 66.42 respectively as summarized in Table 3 along with the biological RI in the different age groups. There were some changes with age in the RI which is quite similar to few other studies worldwide [4, 15]. The 90% confidence intervals corresponding to the reference limits overlapped, indicating that the RIs that applied to the different age groups were, in general not so different from the reference limits of the entire study group. Table 4 gives the comparison of the mean tPSA in different studies done in India [16, 25-27] with our study results in all the age groups. The lower limit of the reference range has increased as the age increased in all the studies and is comparable. Table 5 compares the results obtained in our study and other studies done in different parts of the world [4, 15] for p2PSA, %p2PSA, and PHI. The RI (1.29-12.51) for p2PSA in our study for all age groups combined is similar to the RI (1.88-17.4) in another study done by Wu *et al*., (2019) as detailed in Table -5 [4]. The lower limit of the RI (8.08-66.42)of PHI in our study for all age groups combined is similar to the lower limit of the RI (8.64- 53.37) in another study done by Sun *et al*., (2017) [15] (Table 5).

Table 3: Age-based reference interval (2.5th to 97.5th percentiles) for p2PSA, %p2PSA and PHI

Marker	Age group	n	Percentile	
			2.5th	**97.5th**
p2PSA	**Ages (years)**	406	1.29 (1.25 -1.33)*	12.52 (12.18 – 13.26)*
	< 50	98	1.24	10.27
	50 – 59	182	1.29	11.59
	⩾60	126	3.12	13.44
%p2PSA	**Ages (years)**	406	0.65 (0.59 - 0.70)*	3.82 (3.67 – 3.97)*
	< 50	98	0.57	3.84
	50 – 59	182	0.69	3.71
	⩾60	126	0.62	3.97
PHI	**Ages (years)**	406	8.08 (7.72 - 8.64)*	66.42 (66.17 – 72.69)*
	< 50	98	7.36	65.45
	50 – 59	182	8.29	66.79
	⩾60	126	9.23	72.70

**The 90% confidence interval of the reference limits is given in the bracket only for the RI applicable to the whole study group.*

Table 4: Comparison of mean tPSA in different studies done in India with our study

Age Group (years)	Our study (n=406)	Shenoy *et al.*, (2021) [14] (n=4667)	Karpaghavalli *et al.*, (2020) [25] (n=461)	Gupta *et al.*, (2014) [23] (n=1253)	Agrawal and Karan (2017) [24] (n=1772)
<50	1.16	0.75	0.7	0.65	1.22
50-59	1.77	0.97	0.8	0.79	1.97
60-69	2.89	1.38	1.1	0.88	2.08
70-79	-	1.89	1.04	1.25	-
>80	-	2.19	-	1.45	-

Table 5: Comparison of the age-stratified RI obtained in different studies with our study

Age Groups	Our study (n= 406)	Wu *et al.*, (2019) [4] (n= 726)	Sun *et al.*, (2017) [13] (n= 476)
p2PSA			
Age (years)	1.29-12.52	1.88-17.4	2.22-18.63
< 50	1.24-10.27	2.02-19.02	2.22-6.36
50-59	1.29-11.59	1.60-15.75	8.13-11.74
>60	3.12-13.44	1.28-15.15	7.44-18.63
% p2PSA			
Age (years)	0.65-3.82	1.26-4.54	-
< 50	0.57-3.84	1.17-4.82	-
50-59	0.69-3.71	1.35-3.91	-
> 60	0.62-3.97	1.21-4.41	-
PHI			
Age (years)	8.08-66.42	9.48-45.50	8.64-53.37
< 50	7.36-65.45	9.77-65.28	8.64-46.76
50-59	8.29-66.79	9.98-39.72	22.42-38.52
> 60	9.23-72.70	8.16-40.76	23.96-61.75

Discussion

Interpretation of any laboratory test is invariably based on the RIs. The test values alone are useless unless the lab report has appropriate RI or clinical decision limits. RIs depend on various factors and differences are known to exist between races, ethnic populations, and age groups. Most of the studies on biomarkers of PCa are focused on patients diagnosed with prostate diseases, but in reality, there exists a natural trend of variations in the biomarkers with progressing age among healthy men [14, 15]. This fact needs to be considered while deciding the RIs of these biomarkers to prevent over diagnosis of PCa. To the best of our knowledge, this study represents the first study to establish RI for p2PSA, % p2PSA, and PHI in healthy male Indian population in three different age groups.

Age-group-specific reference intervals for tPSA

Most of the studies across the world [15, 24] and some studies done in India [16, 25-27] have established that tPSA median levels increase with every passing decade and a higher lower reference limit needs to be considered especially after the sixth decade of life. Our study findings are somewhat similar to these studies and the mean tPSA levels were significantly higher in the >60 years age group. The decade-wise comparison of mean PSA in different studies in India is given in Table 4 and the findings of our study for serum tPSA are more comparable to the studies done by Agrawal and Karan [26]. The reason for the slightly higher mean tPSA value when compared to other studies in the < 50 years age group could be because number of subjects. The reasons for the decade-wise increase in serum tPSA levels may be due to physiological or pathological variations in the size of the prostate gland, which is regulated by hormones such as estrogen, insulin, and insulin-like growth factors.

Age-group specific reference intervals for p2PSA, %p2PSA and PHI

For the parameters – p2PSA, %p2PSA, and PHI, our sample size especially in the <50 years age group was restricted to 98 subjects as many of the younger subjects did not undergo a prostate ultrasound and this might be considered as a limitation of this study especially since the CLSI guidelines say that for establishing RIs in any population, it is optimal to include at least 120 subjects [23]. The comparison of the RIs (2.5^{th} to 97.5^{th} percentile) for these biomarkers available in different studies that have focused on age-stratified RI in healthy males [4, 15] with our study is given in Table 5.

Though the RIs show the same trends across the different age groups, they are not the same in all the studies. One of the main reasons for these discrepancies in the studies is because the study by Sun *et al.,* (2017), involving Chinese men of various age groups, used human p2PSA kits manufactured by Elabscience Biotechnology Co. Ltd in Roche E170 Electro Chemiluminescence platform [15]. Again, this is a point to consider that there will be instrument-to-instrument, method-to-method variations possible which can influence the RI, till such a time that harmonization of the calibrators manufactured by various companies is not done. The upper limit of the RI for PHI in our study is much higher when compared to other studies (66.82 vs 45.50 and 53.37) (Table 5). This again reiterates the fact that RI has to be established and validated in different races, ethnicities, or instruments especially when a new biomarker for screening and diagnosis of diseases like cancer is being used.

Though we have proposed these RI for the studied biomarkers for the Indian population, the authors would like to emphasize the fact that using these biomarkers for screening, diagnosis, or prognosis of prostate cancer should be done in conjunction with other diagnostic modalities and use a multimodal approach for clinical interpretation. Moreover, these RIs are useful when the biomarkers have been analysed using Beckman Coulter instruments.

Conclusion

This paper describes the relationship between age and changes in the RIs of the biomarkers PSA, p2PSA, % p2PSA, and PHI and to the best of our knowledge is one of the first studies done in the

Indian population to establish age-stratified reference RIs for these biomarkers. Our study also shows that the upper limit of the RI in Indian males for PHI was higher than found in studies done in other races and ethnicities.

Limitations

This study also has certain limitations concerning the sample size, as we could not meet the required number of samples during the study period in the younger age group of less than 50 years, which would have added to the validity of this study. Another limitation of this study is that we have not calculated separate RIs for the age group 40 to 50 years and clubbed all subjects who were less than 50 years.

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